

BASIC SCIENCE

Inflammation-Induced Vasoconstrictor Hyporeactivity Is Caused by Oxidative Stress

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OBJECTIVES	We sought to determine the role of oxidative stress in the development of vascular dysfunction in inflammation.
BACKGROUND	Hyporeactivity to catecholamines and other vasoconstrictors is present in acute inflammation. Because oxidative stress plays a significant role in inflammation, impaired responsiveness may be overcome by anti-oxidants.
METHODS	In randomized, double-blind, cross-over studies, forearm blood flow (FBF) responses to norepinephrine (NE), angiotensin II (ANG II), and vasopressin (VP) were assessed before and 4 h after induction of systemic inflammation by low doses of <i>Escherichia coli</i> endotoxin (lipopolysaccharide [LPS], 20 IU/kg intravenously) or after placebo in healthy volunteers. Furthermore, the effect of intra-arterial vitamin C (24 mg/min) or placebo on NE-induced or ANG II-induced vasoconstriction was studied after LPS.
RESULTS	Administration of LPS caused systemic and forearm vasodilation, increased white blood cell count, elevated body temperature, and reduced vitamin C plasma concentrations. Lipopolysaccharide decreased the responses of FBF to NE by 59%, to ANG II by 25%, and to VP by 51% (n = 9, p < 0.05, all effects). Co-administration of vitamin C completely restored the response to NE and to ANG II, which was comparable to that observed under baseline conditions (n = 8).
CONCLUSIONS	<i>E. coli</i> -endotoxemia reduces FBF responsiveness to vasoconstrictors. The hyporeactivity can be corrected by high doses of vitamin C, suggesting that oxidative stress may represent an important target for inflammation-induced impaired vascular function. (J Am Coll Cardiol 2003;42:1656-62) © 2003 by the American College of Cardiology Foundation

Despite advances in treatment, sepsis is still associated with high mortality (1). Persistent systemic vasodilation requires administration of catecholamines to increase blood pressure (BP) and perfuse tissues. Improvement of hypoperfusion in sepsis is limited by hyporeactivity to exogenously administered catecholamines such as norepinephrine (NE). In a previous study, we demonstrated systemic and regional vascular hyporeactivity to adrenoceptor agonists after ad-

hyporesponsiveness extends to other vasoconstrictors during acute systemic inflammation induced by *E. coli* endotoxin in humans. The purpose of the present study was to test if the vasoconstrictor potency of angiotensin II (ANG II) and vasopressin (VP) is also reduced during inflammation, and if endotoxin-associated hyporesponsiveness to vasoconstrictors can be restored by high doses of the anti-oxidant vitamin C. Systemic inflammation was generated by administration of low doses of lipopolysaccharide (LPS) in healthy volunteers (2,5,6), and forearm vascular function was studied using strain-gauge plethysmography.

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ministration of low doses of *Escherichia coli* endotoxin in humans (2). This adrenoceptor hyporesponsiveness was detectable in the absence of increased nitric oxide (NO) bioactivity. The mechanisms underlying this hyporeactivity are unclear, but oxidative stress secondary to increased production of reactive oxygen species (ROS) has been implicated in animal experiments (3). Increased ROS generation is present in inflammation, and these highly reactive species can inactivate biologic mediators, including catecholamines (4). Furthermore it is not known if vascular

METHODS

The study protocols were approved by the Ethics Committee of the University of Vienna and comply with the Declaration of Helsinki, including current revisions and the Good Clinical Practice guidelines.

Study population. Twenty-five healthy male subjects between 21 and 38 years of age, from whom informed consent was obtained before enrollment, were included in one of three protocols. Where indicated, subject randomization was done after enrollment by a member of the Department of Clinical Pharmacology not involved in the study. All subjects were given a complete health examination (including physical examination, electrocardiogram, and laboratory screening) within 14 days before the first study day. All

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Abbreviations and Acronyms

ANG II	= angiotensin II
ANOVA	= analysis of variance
BP	= blood pressure
FBF	= forearm blood flow
LPS	= lipopolysaccharide (<i>E. coli</i> endotoxin)
NE	= norepinephrine
NO	= nitric oxide
NOS	= nitric oxide synthase
NO _x	= nitrite
O ₂ ^{•−}	= superoxide radicals
ROS	= reactive oxygen species
VP	= vasopressin

subjects were non-smokers and had no history or signs of arterial hypertension, hypercholesterolemia, or other cardiovascular risk factors. All of the subjects claimed not to have ingested any prescribed medications, or over-the-counter drugs from two weeks before screening until the study was complete. After an overnight fast, studies were conducted in a quiet room with an ambient temperature of 22°C with full resuscitation facilities.

Generation of systemic inflammation in vivo. Twenty IU/kg body weight LPS (dose corresponding to 2 ng/kg; National Reference Endotoxin, *E. coli*, U.S.P. United States Pharmacopeia Convention Inc., Rockville, Maryland) was administered intravenously as a bolus infusion to induce acute inflammation. Injection of LPS to humans has been established by other groups and at our institution as a model for acute systemic inflammation (2,5,6). It has been demonstrated that this dose of LPS impairs the vascular responses to adrenergic vasoconstrictors and endothelium-dependent vasodilators, with maximum clinical effect and vascular hyporeactivity approximately 4 h after LPS administration (2,6). The flu-like symptoms are mild and transient, and subjects can be discharged after approximately 8 to 10 h in good health. Blood for analysis of white blood cell count was drawn at baseline and 4 h after LPS administration. Tympanic temperature (Thermoscan pro, Braun AG, Germany) was measured at frequent intervals; electrocardiogram, pulse rate, and BP were recorded with an automated device (Hewlett Packard CMS patient monitor, Palo Alto, California).

Forearm blood flow measurements. Forearm blood flow (FBF) was measured as described previously (2,7). Briefly, strain gauges were placed on the forearms and connected to plethysmographs (EC-6, DE Hokanson Inc., Bellevue, Washington) to measure changes in forearm volume in response to inflation of venous congesting cuffs. Bilateral plethysmography was used, expressing drug effects as the ratio of blood flow in the intervention to the control arm (2,8), where baseline ratio was defined as 100%. Wrist cuffs were inflated to suprasystolic pressures during each measurement to exclude circulation to the hands. Flow measurements were recorded for 9 s at 30-s intervals during drug

infusion. Traces were analyzed using the NIVP3 software (Version 5.25, DE Hokanson Inc.).

Effect of vasoconstrictors. In a double-blind study, vascular responses to NE, ANG II, and VP were measured in nine subjects (27 ± 5 years) on two different trial days. The order of the two days was randomized. On one day LPS was administered, and on the other day a placebo vehicle, with a washout period of at least seven days. Vasoconstrictors were administered intra-arterially on both trial days. The sequence of the vasoconstrictors under study was randomized between subjects but remained identical within each subject.

A fine-bore needle (27G needle Sterican, B. Braun, Melsungen, Germany) was inserted into the brachial artery of the non-dominant arm for infusion of the vasoconstrictors. After a 20-min resting period, baseline FBF measurements were made in response to increasing intra-arterial doses of NE (60, 120, 240 pmol/min; Arterenol, Aventis, Strasbourg, France), ANG II (25, 50, 100 pmol/min; Clinalfa, Läufelfingen, Switzerland), and VP (3.2, 6.4, 16 ng/min; Clinalfa). Each dose was infused for 5 min, and a washout period established control blood flow between drugs under study. After dose-response curves had been constructed, a systemic intravenous bolus of LPS (20 IU/kg) or placebo (0.9% NaCl) was given. The FBF responses to vasoconstrictors were repeated 240 min later, as described earlier.

Effects of vitamin C. Two separate studies were conducted in 16 volunteers (26 ± 3 years) to study the effect of vitamin C, which followed a double-blind, randomized, cross-over design with a washout period of at least seven days. In these experiments, the order of the study days (LPS with or without co-administration of vitamin C) was randomized. In the first study ($n = 8$), a dose-response curve of FBF to NE (60, 120, 240 pmol/min) was constructed before and 4 h after LPS on two different trial days; 230 min after LPS, subjects received a continuous intra-arterial infusion of vitamin C (24 mg/min, Mayerhofer GmbH, Linz, Austria) (9,10) or physiologic saline over 25 min on two different days. The response of FBF to NE co-administered with vitamin C or placebo was assessed 10 min after the start of infusion.

In the second study ($n = 8$), FBF measurements were made in response to intra-arterial ANG II (25, 50, 100 pmol/min) before and 4 h after LPS on two different trial days. Again, an intra-arterial infusion of vitamin C or physiologic saline was administered 230 min after LPS, and the response to ANG II co-administered with vitamin C or placebo was assessed 10 min after the start of infusion, as previously described. Plasma for quantification of vitamin C levels (Hitachi 911, Roche, Basel, Switzerland) was collected from the non-intervention arm at baseline and 4 and 8 h after LPS.

Control experiments. To study the direct effects of vitamin C on NE-induced and ANG II-induced vasoconstriction, control experiments with vitamin C alone, without

Table 1. Systemic Hemodynamics, Laboratory Parameters, and Forearm Blood Flow

	Baseline	4 h After LPS
Heart rate (beats/min)	63 ± 2	80 ± 2†
Systolic blood pressure (mm Hg)	127 ± 2	122 ± 3*
Diastolic blood pressure (mm Hg)	70 ± 2	63 ± 1†
Body temperature (°C)	36.3 ± 0.1	37.8 ± 0.1†
White blood cell count (G/l)	5.4 ± 0.3	9.5 ± 0.6†
Forearm blood flow (ml/min/100 ml)	3.9 ± 0.1	5.0 ± 0.2*

Data are pooled for 25 subjects and presented as means ± SEM. * $p < 0.05$, † $p < 0.01$ versus baseline (paired t test).

LPS = lipopolysaccharide.

previous LPS administration, were performed on an open-label trial day in eight subjects. The schedule of this study day was otherwise identical to that described previously.

Statistical analysis. All data sets were tested for normal distribution by the Kolmogorov-Smirnov test. Data for white blood cell count, vitamin C, and FBF were normally distributed. Data for temperature, pulse rate and BP were log-normal distributed and therefore log transformed, after which they tested positive for normal distribution. Hemodynamic and laboratory parameters were expressed as absolute values or percent changes from baseline and compared using Student paired or unpaired t test. The FBF was expressed as ml/min/100 ml forearm volume. Period and carry-over effects for the main outcome parameters were assessed with Student t test or the Wilcoxon rank-sum test, depending on data distribution. The effects of NE, ANG II, and VP at baseline and after LPS or placebo administration were assessed by analysis of variance for repeated measurements (ANOVA), followed by Bonferroni corrected t tests, using the Statistica software package (Release 5.0, StatSoft Inc., Tulsa, Oklahoma). A value of $p < 0.05$ was considered statistically significant. Values are presented as means ± SEM unless indicated otherwise.

RESULTS

Systemic hemodynamics, resting FBF, and laboratory parameters were comparable between trial days at baseline (not shown). There was no evidence of a period effect or carry-over between the first and the second trial day in cross-over experiments. All infusions were well tolerated, and no adverse events were reported. After LPS, the expected mild and transient flu-like symptoms occurred. The LPS increased white blood cell count after 4 h ($p < 0.05$, Table 1), which was paralleled by a decrease in BP and an increase in pulse rate ($p < 0.05$, Table 1). Hemodynamic parameters returned to baseline 8 h after LPS. Mean FBF also increased significantly after LPS ($p < 0.01$, Table 1).

Effects of vasoconstrictors after LPS. At baseline, NE, ANG II, and VP caused a dose-dependent decrease in FBF to $72 \pm 3\%$, $55 \pm 5\%$, and $79 \pm 3\%$ of control values, respectively ($p < 0.05$, all drugs). Four hours after LPS administration, responses to all vasoconstrictors were attenuated: NE reduced FBF to a maximum of $89 \pm 3\%$, ANG II to $66 \pm 5\%$, and VP to $90 \pm 3\%$ of control values (Fig.

1). The ANG II-induced vasoconstriction was, however, reduced to a lesser extent compared with the other vasoconstrictors ($p < 0.05$). Administration of placebo had no effect on the vasoconstrictors; FBF was reduced by NE to $70 \pm 3\%$, by ANG II to $53 \pm 4\%$, and by VP to $75 \pm 3\%$ of control values, which was not different from baseline measurements (Fig. 1, $p = \text{NS}$).

Effect of vitamin C on NE and ANG II responsiveness.

Norepinephrine decreased FBF by $25 \pm 3\%$ at baseline, which was attenuated after LPS to $8 \pm 10\%$ ($p < 0.05$). This hyporeactivity was completely restored by co-administration of vitamin C (Fig. 2). Angiotensin II decreased FBF by $34 \pm 6\%$ at baseline and by $22 \pm 5\%$ after LPS ($p < 0.05$). Again, co-infusion of vitamin C restored the vasoconstrictor potency of ANG II (Fig. 3). Systemic vitamin C concentrations decreased by 29% after LPS ($p < 0.05$) and increased approximately sixfold during local administration of vitamin C in the contralateral arm ($p < 0.001$, Table 2). Vitamin C had no effect on systemic hemodynamics, baseline FBF, or NE- or ANG II-induced vasoconstriction in control experiments (Table 3).

DISCUSSION

We have demonstrated that vasoconstrictor hyporeactivity during *E. coli* endotoxemia in humans is not specific for adrenoceptor agonists but also occurs in response to ANG II and VP. Further, this study provides evidence for a direct link between endotoxemia with impaired vasoconstrictor function and oxidative stress, because the response to NE and ANG II was normalized by co-administration of the anti-oxidant vitamin C. Oxidative stress with consumption of anti-oxidants and inactivation of vasoconstrictors may play a significant role in the development of hyporeactivity to exogenous vasoconstrictors during endotoxemia. The functional alterations underlying impaired vasoconstrictor responsiveness are not fully understood so far. Overproduction of NO by the inducible isoform of the nitric oxide synthase (NOS) has been well characterized in animal experiments. Assessment of NO formation proves difficult in vivo, because its half-life is very short. Indirect methods are influenced by many confounding factors. Measurement of nitrate and nitrite (NOx), the stable metabolites of NO in blood and urine, has been repeatedly used (11). However, the concentrations of NOx vary considerably even in healthy subjects (12), and the source of NO cannot be derived. Exhaled NO as a marker of airway NO production is of limited value in predicting NO production in the peripheral vascular bed during systemic inflammation. A change in exhaled NO concentrations in endotoxin-challenged volunteers was not paralleled by alterations in systemic hemodynamics (11).

In our model of systemic inflammation, we have not detected increased NOS activity, either by NOS messenger ribonucleic acid expression in leukocytes or increased responsiveness to NOS inhibitors (2). Consistent with this is

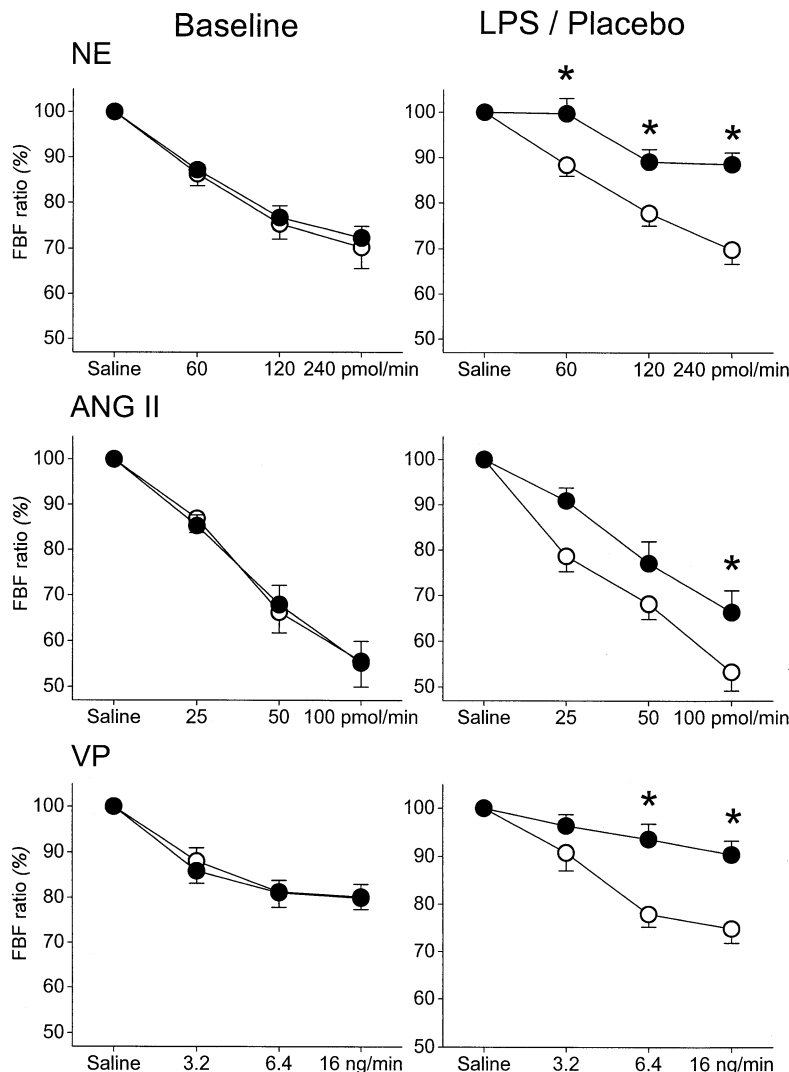


Figure 1. Forearm blood flow (FBF) dose-response curves to norepinephrine (NE, upper panel), angiotensin II (ANG II, middle panel), and vasopressin (VP, lower panel) at baseline and after administration of LPS (closed symbols) or placebo (open symbols). The response to vasoconstrictors was attenuated after LPS. The FBF ratio (intervention vs. control arm, baseline defined as 100%) is expressed as mean \pm SEM. $n = 9$; * $p < 0.05$ between groups, repeated measures ANOVA and Bonferroni corrected t tests.

the lack of beneficial effects of NOS inhibitors in human sepsis (3,13,14). NO may have a role in certain types of bacterial sepsis in humans, particularly in the presence of putrefaction (15).

Alternative vasoconstrictors have been proposed for the treatment of systemic hyporeactivity in sepsis (16–20), and we set out to measure vascular responses to ANG II and VP accordingly. However, the vasoconstrictor potency of all drugs under study was significantly decreased, which is in accordance with previous animal studies (21–23). It has been demonstrated that the final common pathway of the vasoconstrictors, that is, increase of intracellular Ca^{2+} , could be influenced by LPS directly (24,25) and reduce the responsiveness to vasoconstrictors (26). However, there are no data from human studies available so far on the role of altered Ca^{2+} handling and inflammation. In previous experiments, we observed that LPS administration did not

alter the response to the NOS inhibitor *N*-monomethyl-L-arginine (2), arguing against a major effect of LPS on the vascular smooth muscle contractile apparatus in this experimental model.

Hyporesponsiveness to NE during systemic inflammation is a consistent finding in experimental studies (16,27,28) and was confirmed in this setting. It has been suggested that increased generation of ROS like superoxide radicals (O_2^-) in inflammation would exceed the capacity of the endogenous anti-oxidant defense system with consumption of anti-oxidants (14). As a corollary, a significant decrease in circulating vitamin C concentrations was detectable in our experiments. At present, there is no method available to assess O_2^- formation in vivo in humans directly. However, there is evidence that O_2^- can inactivate NE during inflammation, as autoxidation of catecholamines could be prevented by a superoxide dismutase mimetic, which me-

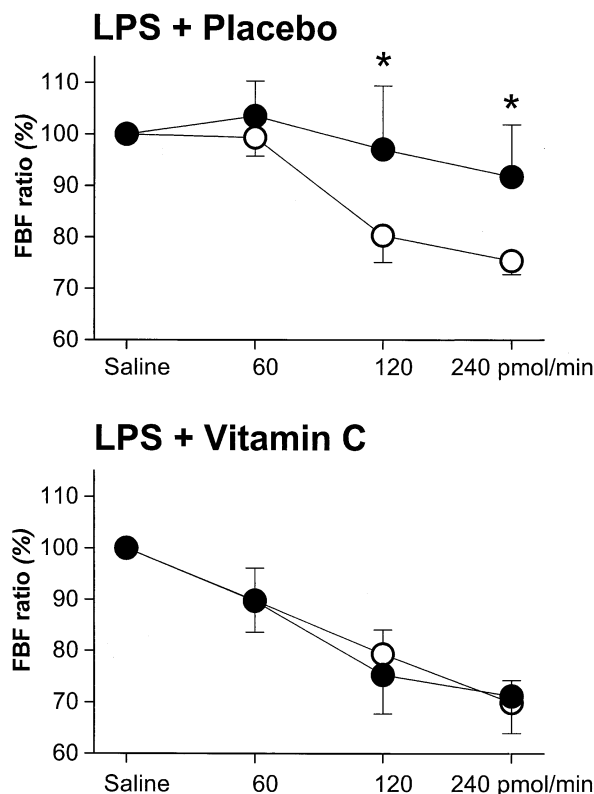


Figure 2. Vasoconstriction to norepinephrine at baseline (open symbols) and 4 h after LPS, with co-infusion of placebo (closed symbols, upper panel) or vitamin C (closed symbols, lower panel) on two different study days. The FBF ratio (intervention vs. control arm, baseline defined as 100%) is expressed as mean \pm SEM. $n = 8$; $p < 0.05$ between groups, repeated measures ANOVA and Bonferroni corrected t tests.

tabolizes O_2^- (3). Vitamin C is an effective scavenger of reactive species at this high local concentration (29) and has been used to assess the bioactivity of endogenous O_2^- in biologic systems (30). In the present study vitamin C restored hyporeactivity to NE and ANG II caused by LPS, and had no effect on vascular reactivity in subjects not exposed to LPS.

Likewise, hyporeactivity to ANG II after LPS was also restored by vitamin C. There is evidence that the aromatic tyrosine residue of ANG II can be modified by peroxynitrite, the product of the reaction of NO and O_2^- (31,32). In animal experiments, peroxynitrite reduced the vasoconstrictor effect of ANG II accordingly (31). Considering the continuous flux of newly synthesized NO by the vascular endothelium (2,9), it can be speculated that nitrosation and inactivation of circulating ANG II could occur at sites with excess O_2^- production. This is compatible with in vitro data which demonstrate that vitamin C is an effective quencher of O_2^- and also of peroxynitrite (33,34). The chemical reaction between ANG II and ROS is more likely to contribute to impaired reactivity than downregulation of angiotensin receptors, which can be detected later in sepsis (22). One would therefore expect that the efficacy of ANG II is diminished further after longer periods of inflammation than in our LPS model.

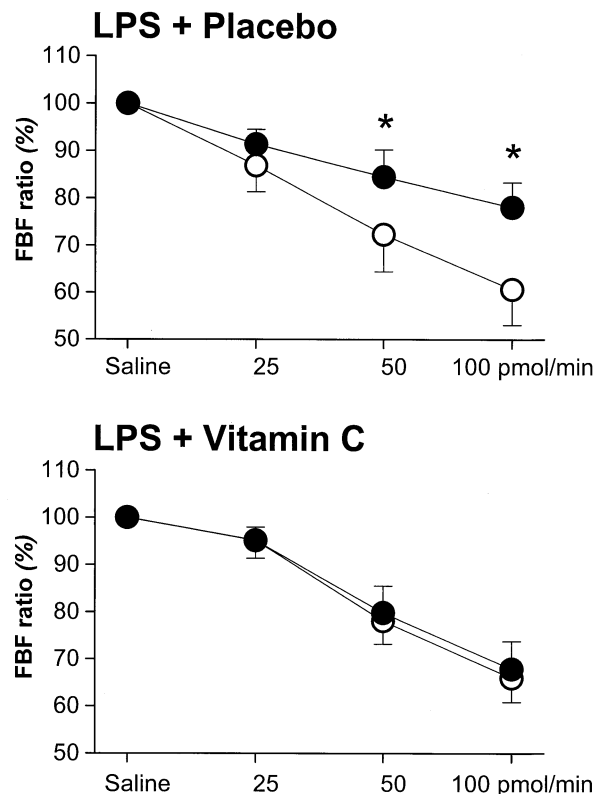


Figure 3. Vasoconstriction to angiotensin II at baseline (open symbols) and 4 h after LPS, with co-infusion of placebo (closed symbols, upper panel) or vitamin C (closed symbols, lower panel) on two different study days. The FBF ratio (intervention vs. control arm, baseline defined as 100%) is expressed as mean \pm SEM. $n = 8$; $p < 0.05$ between groups, ANOVA and Bonferroni corrected t tests.

The results obtained for VP have to be interpreted with caution, because VP exerts a biphasic response in the forearm with vasodilation at higher doses (35). Nevertheless, these experiments demonstrate that VP is also subject to reduced vasoconstrictor potency after LPS. The octapeptide VP also contains the aromatic tyrosine and phenylalanine residues, which may react with reactive oxygen or nitrogen species rendering the peptide inactive. These findings argue for a crucial role of the oxidative status in the development of vasoconstrictor dysfunction in endotoxemia.

In sepsis, clinical trials have suggested that VP or analogues restore BP in patients with catecholamine-resistant septic shock (18–20). However, in these studies VP was given to patients to whom NE had already been adminis-

Table 2. Systemic Vitamin C Plasma Concentrations at Baseline and 4 and 8 h After LPS

	Vitamin C Concentration ($\mu\text{mol/l}$)		
	Baseline	LPS, 4 h	LPS, 8 h
Vitamin C	63.5 \pm 7.4	356.2 \pm 23.3*†	176.5 \pm 19.5*†
Placebo	64.3 \pm 3.9	45.4 \pm 5.0*	68.4 \pm 9.9

Vitamin C (24 mg/min) or placebo was infused over 25 min into the brachial artery, 230 min after lipopolysaccharide (LPS) administration, on different study days. Data are presented as means \pm SEM ($n = 16$). * $p < 0.05$ versus baseline (paired t test); † $p < 0.05$ between study days (unpaired t test).

LPS = lipopolysaccharide.

Table 3. FBF (%) in Response to Increasing Doses of Norepinephrine and Angiotensin II at Baseline and With Co-Infusion of Vitamin C

	Baseline	Vitamin C
Norepinephrine		
60 pmol/min	94.7 ± 6.6	83.7 ± 4.3
120 pmol/min	86.5 ± 6.4	83.1 ± 6.3
240 pmol/min	70.5 ± 6.6	62 ± 5.2
Angiotensin II		
25 pmol/min	85.8 ± 2.9	81.0 ± 4.1
50 pmol/min	70.2 ± 4.9	69.7 ± 3.7
100 pmol/min	58.4 ± 5.3	56.5 ± 4.7

Data are presented as means ± SEM, n = 8. Vitamin C had no effect on forearm blood flow (FBF) responsiveness.

tered, and it is not certain to what extent the response to VP was dependent on background NE, rather than evidence of preserved sensitivity to VP.

One possible limitation of this cross-over study is that the results could be confounded by endotoxin tolerance, as seen in vitro and in animal studies (36). In the present study and in our previous study, however, no hemodynamic tolerance to repeated administration of LPS was observed in healthy volunteers when there was a minimum washout period of seven days between LPS administrations (9). This might be due in part to the rather low dose of LPS used compared with animal and in vitro experiments. In humans, endotoxin tolerance has been described in ex vivo stimulation of whole blood early after previous systemic LPS administration (37) or in veins after daily local instillation of high LPS doses (28).

In summary, we have demonstrated that hyporeactivity to vasoconstrictors during inflammation is not exclusive for catecholamines and involves ANG II and VP in vivo. Secondly, inflammation is associated with consumption of anti-oxidants, and hyporeactivity to vasoconstrictors caused by LPS can be reversed by high circulating concentrations of vitamin C. These observations suggest that administration of vitamin C might restore sensitivity to constrictors in clinical endotoxemia.

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